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SMUFD D/A ltr, 14 Feb 1972

TRANSLATION NO. 1254

DATE: 11 Dec 1964

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

BIOCHEMICAL STUDIES ON RICE BLAST DISEASE

Translation of an article by Hiroshi Otsuka, Kinjiro Tamari, Nagahiro Ogasawara and Hondaryoji in the Japanese-language journal Journal of the Agricultural Society of Japan, Vol. 32, No. 11, 1958, pp. 893-897.

PART X

The Biochemical Classification of Piricularia Oryzae Cavara (6)

On the Production of Nicotinic Acid by Piricularia Oryzae Cavara

Previously we conducted tests on the function of biotin as a growth element in Piricularia Oryzae (1) and we established that out of 45 strains, two grew in a biotin-free synthetic culture medium, and that practically no biotin was produced. Extensive research has been conducted not only on biotin as a growth element in piricularia oryzae, but also on thiamine, and a long time ago, Ito, et. al. (2) established excellent results for oryzanine in stimulating the development and formation of cells. F.W. Leaver (3) recognized that thiamine is an absolute requirement for the growth of bacteria and Tanalka et. al. (4) recognized that, except for biotin, thiamine is necessary as a supplement to the normal growth of piricularia oryzae. Likewise, Otani (5) studied the state of growth of bacteria in a culture medium to which biotin and thiamine were added and in media free of these substances and found that thiamine is a supplementary element.

As a result of our previous study on which of 47 strains would grow in a thiamine-free synthetic culture medium, we established that 9 strains would grow well and that 38 strains required thiamine. We also observed that among the strains requiring thiamine, excellent growth of strains occurred in an artificial culture medium to which nicotinic acid was added instead of thiamine.

By means of a bioassay employing lactobacillus arabinosus and leuconostoc mesenteroides, we confirmed that nicotinic acid is produced in the culture filtrate of strains which grow rather well in a thiamine-free medium which were cultivated, in a synthetic, thiamine-free culture medium. G. W. Keitt (6) has obtained interesting results in a study of the relationship between the pathogenicity and the requirement of biochemical mutants of Venturia inaequalis for microscopic substances such as nicotinic acid and others. C. Yanofsky (7) has done some detailed research on the production of nicotinic acid in microorganisms, especially molds, but few other examples for molds can be found. We found this point of interest and consider it worthy of further investigation.

Experiment

(1) Piricularia oryzae tested. 47 strains of piricularia oryzae received from the National Institute of Agricultural Sciences as in the previous report.

(2) Previous culture medium. As in Report 10 (No. I).

(3) Thiamine-free synthetic culture medium. The constituents of the medium are as in Report 10 (No. IV) without thiamine, but with 5 γ/l of biotin added. The conditions of the culture are the same as in Report 10 (no. IV).

(4) Synthetic culture medium with addition of nicotinic acid: 1 mg/l of nicotinic acid added to the above thiamine-free medium.

(5) Determination of quality of growth. As in Report 10 (No. IV).

(6) Determination of nicotinic acid. Determination was made by bioassay using lactobacillus arabinosus ATCC 8014 and leuconostoc mesenteroides ATCC 8042 for establishing the absence of nicotinic acid in the culture medium and for estimating the amount of nicotinic acid. The bioassay procedure was titration with 1/20 N of NaOH in the culture liquid (2ml) after maintaining it at 37° for 72 hours, which is the normal procedure. The culture constitutents and the standard curves for the bioassay are given respectively, in Table 2 and Fig. 1.

We put 30 ml each of thiamine-free culture medium into 100 ml Ehrlenmeyer flasks (absence of thiamine determined by bioassay with Lactobacillus fermenti ATCC 9338) and after pressure-killing the bacteria for ten minutes at ten pounds, nine strains which grew relatively well in thiamine-free medium were added to this culture medium and cultivated at 25° for 14 days.

TABLE 1. STATE OF GROWTH OF BACTERIA IN THIAMINE-FREE SYNTHETIC CULTURE MEDIUM TO WHICH NICOTINIC ACID HAS BEEN ADDED

Weight of Mycelia g x 200

Strain	Complete medium	Thiamine-free medium	Medium	Strain	Complete medium	Thiamine-free medium	Medium
5414	2.65	0.25	2.10	5517	3.20	0.50	1.05
5418	3.25	0.40	1.95	5518	—	—	—
3	2.80	1.60	1.30	5519	2.80	0.55	1.30
No. 1	2.10	1.85	3.00	5520	2.23	0.15	0.20
No. 2	3.60	2.40	2.60	5521	2.50	1.30	1.40
No. 11 F8 hetero	2.20	0.70	0.65	5522	3.65	0.35	2.30
No. 11 hetero	3.10	0.70	1.20	5523	3.50	0.60	0.60
No. 188 hetero	2.50	0.20	0.30	5524	2.65	0.05	0.20
P_a	3.05	0.20	1.05	5525	3.00	0.10	1.75
A25	3.50	0.40	2.10	5526	3.40	0.10	1.10
A30	2.80	1.00	1.75	5527	2.50	2.40	1.00
5309	3.10	0.20	0.60	5528	2.50	0.50	1.00
5311	3.30	1.35	1.80	5529	3.70	2.20	2.35
5327	3.70	0.30	1.95	5532	3.00	0.10	1.50
5330	2.40	2.15	2.45	5533	3.20	0.60	0.50
5333	2.70	0.50	0.80	5534	3.40	1.25	1.30
5404	2.65	0.70	0.70	5535	3.60	1.80	2.65
5420	2.80	0.05	0.10	5536	3.70	1.00	1.60
5424	2.20	0.05	0.05	5537	3.50	0.20	1.00
5425	3.05	0.10	0.85	5539	2.60	0.20	0.25
5415	2.30	0.25	1.85	5540	3.90	0.75	0.50
5514	2.30	0.80	1.45	CO-home	3.00	2.10	2.00
5515	3.70	2.00	1.95	SO-home	2.60	2.00	2.45
5516	2.90	1.20	2.30				

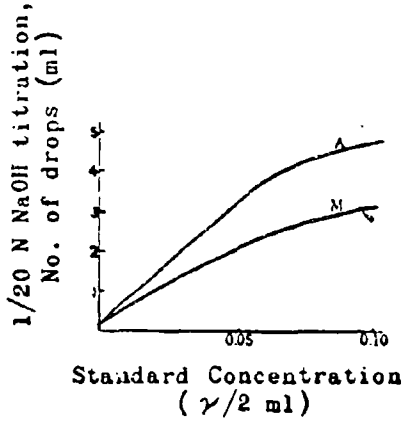


Figure 1. Standard Curves for Nicotinic Acid.

A. Lact. arabinosus ATCC 8014
M. Leuc. mesenteroides ATCC 8042.

TABLE 2. CONSTITUENTS FOR BASIC CULTURE MEDIUM FOR BIOASSAY FOR NICOTINIC ACID (DOUBLE STRENGTH)

Vitamin-free casein hydrolizate (T.N.1.28%) 10% solution 10 ml

Glucose	4g	Folic acid	2 γ
Na-acetate	4g	Adenine-H_2SO_4	2mg
L-systine	20mg	Guanine-HCl	2mg
DL-tryptophane	20mg	urasyl	2mg
Thiamine-HCl	200γ	xanthin	2mg
Riboflavin	200γ		
Pyridoxal-HCl	200γ	A-solution*	2ml
Pyridoxine-HCl	200γ	B-solution**	2ml
p-Aminobenzoic acid	60γ	Distilled water to make (pH 6.8) total.	100 ml [illegible]
Ca-Pantothenate	200γ		
Biotin	2γ		

*Solution A. 5g of KH_2PO_4 and 5g of K_2HPO_4 plus enough distilled water to make 100 ml.

**Solution B. 2 g of $MgSO_4.7H_2O$; 0.1g of $FeSO_4.7H_2O$; 0.1g of $MnSO_4.4H_2O$; 0.1 g NaCL plus enough distilled water to make 100 ml.

Table three shows the results of weighing the mycelia which were grown, after they were dried at 100°, and the results of determination by bioassay of the nicotinic acid in the culture filtrate. Strains which require thiamine and in which nicotinic acid cannot be substituted were placed in 100 ml Ehrlenmeyer flasks. The bacteria were killed by partially adding a complete synthetic medium to which 30 ml of thiamine was added, the resulting combination was cultivated for 14 days at 25° and then the weight of the dried mycelia was determined. A bioassay was made of the amount of nicotinic acid generated in the culture filtrate. The results of the above are shown in Table 4.

Table 5 shows the results of bioassays of the amount of nicotinic acid produced in the culture filtrate, and the weight of the dried mycelia measured at different stages in time for strain No. 1 provided by Hokkaido University and for the S0-homo strain provided by the Agriculture Faculty of the Tokyo University of Agriculture and Technology, which were cultivated in a thiamine-free culture medium and which produced nicotinic acid and were then cultivated at 25° in a thiamine-free culture medium.

TABLE 3. WEIGHT OF MYCELIA IN THIAMINE-FREE SYNTHETIC MEDIUM AND YIELD OF NICOTINIC ACID.

Strain	Weight of mycelia	a. nicotinic acid produced (γ/ml) Leuc. mesenteroides	Recovery (%)	b. amount of product analogous to nicotinic acid (by L. arabinosus) (γ/ml)	Recovery (%)
Control*	—	0.0	100.0	0.0	99.0
No. 1	0.0960	2.91	96.9	2.21	95.9
No. 2	0.2210	0.74	110.6	0.65	102.4
5330	0.0630	0.17	95.7	0.19	120.7
5515	0.1200	0.35	94.5	0.41	100.2
5527	0.1450	0.18	93.3	0.25	92.9
5535	0.1550	0.37	89.5	0.37	96.6
CO-homo	0.1660	0.29	94.5	0.32	97.0
SO-homo	0.1920	0.89	99.3	0.81	97.2

*Control is thiamine-free culture medium in which no bacteria were planted.

TABLE 4. WEIGHT OF MYCELIA IN COMPLETE CULTURE MEDIUM AND YIELD OF NICOTINIC ACID

Strain	Weight of mycelia	a. nicotinic acid produced (γ/ml) Leuc. mesenteroides	Recovery (%)	b. amount of product analogous to nicotinic acid (by L. arabinosus) (γ/ml)	Recovery (%)
Control	—	0.0	99.5	0.0	100.0
3	0.1230	0.37	111.7	0.42	98.9
No. 11 F8 hetero	0.1910	0.60	109.8	0.54	97.2
No. 11 hetero	0.1810	0.51	101.3	0.53	96.4
P_2	0.1865	0.93	96.4	0.79	99.2
5309	0.1930	0.14	118.9	0.20	104.6
5333	0.1950	0.20	104.0	0.19	119.8
5404	0.1860	0.46	112.8	0.44	100.2
5420	0.1560	1.16	99.4	1.03	99.3
5424	0.1800	0.60	96.4	—	—
5425	0.1760	0.1	107.4	0.16	97.3

Table 4 (Continued):

5514	0.1570	1.43	96.7	1.37	87.3
5517	0.1600	2.64	108.5	2.21	103.3
5519	0.1660	0.64	99.1	0.62	96.0
5520	0.1220	0.19	105.5	0.21	101.2
5526	0.2110	0.15	94.9	0.16	88.4
5526	0.1550	1.16	101.9	1.17	93.4
5532	0.1650	0.89	94.0	–	–
5533	0.1550	0.12	105.7	0.11	94.6
5537	0.1580	0.94	98.6	0.84	91.0
5539	0.1600	0.44	102.1	0.48	95.5

TABLE 5. WEIGHT OF 2 STRAINS AT DIFFERENT STAGES IN TIME AND AMOUNT OF NICOTINIC ACID PRODUCED IN THIAMINE-FREE CULTURE MEDIUM

No. of days cultivated	Weight of mycelia (g x 200)	a. Nicotinic acid produced (γ/ml) Leuc. mesenteroides	Recovery (%)	b. Amount of product analogous to nicotinic acid (by L. Arabinosus) (γ/ml)	Recovery (%)
4 days	0.05	0.08	101.4	0.04	99.1
8 days	1.20	0.15	88.3	0.08	95.8
12 days	2.70	0.30	78.3	0.30	97.3
15 days	4.00	0.63	79.8	0.67	85.0
19 days	3.00	2.56	82.2	2.88	106.0
22 days	2.80	2.36	67.8	3.55	104.5
SO-homo					
4 days	0.05	0.0	101.5	0.10	101.8
8 days	1.10	0.0	98.0	0.15	102.6
12 days	2.50	0.25	91.1	0.23	106.7
15 days	3.36	0.26	96.9	0.30	100.0
19 days	2.70	0.90	95.9	1.02	105.3
22 days	3.46	3.12	110.9	3.35	95.0

Considerations.

As a result of observing the state of cultivation of piriculoria oryzae bacteria which were planted in a thiamine-free synthetic culture medium, it was found that generally the growth of the bacteria in the thiamine-free culture medium was poor in most cases. However, 9 strains, including strains Nos. 1 and 2 from Hokkaido University, strains 5330, 5515, 5527, 5529, and 5535 from the National Institute of Agricultural Sciences, and strains CO-homo and SO-homo from the agricultural faculty of the Tokyo University of Agriculture and Technology, grew comparatively well in a synthetic, thiamine-free culture medium.

It is possible to classify into groups which grow in a thiamine-free synthetic culture medium and groups which will not grow in such a

medium, but this fact indicates that there is considerable difference in the amount of thiamine required, and the above nine strains hardly require any thiamine at all. CO-homo and SO-homo require biotin (S).

Next, as a result of studying the conditions of cultivation when the bacteria were grown in a thiamine-free synthetic culture medium to which 1mg/l of nicotinic acid was added, it was found that reproduction is in general poor and that nicotinic acid cannot be substituted for thiamine. However, eight strains, Nos. 5414, 5418, 5327, 5415, 5516, and 5522 of the National Institute of Agricultural Sciences and strains A25 and A36 of the Chugoku Agricultural Test Station reproduced rather well in a culture medium to which nicotinic acid was added, and the other strains absolutely require thiamine. Thus, it is completely unclear under what procedure nicotinic acid can be substituted for thiamine, but we can surmise that there is some relationship between thiamine and nicotinic acid.

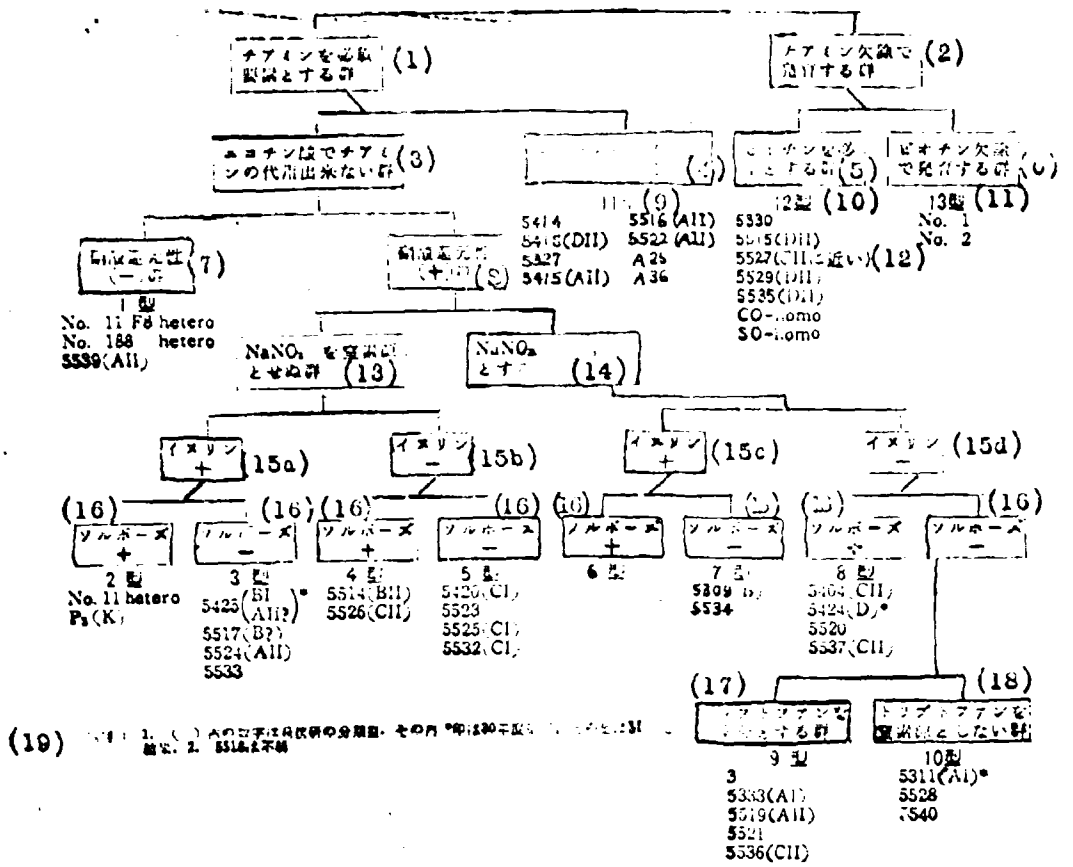
In order to understand the above more extensively, groups which did not require thiamine were cultivated in a thiamine-free synthetic culture medium and their state of growth observed. Also, a bioassay was made to determine whether nicotinic acid was produced in the culture filtrate. W.A. Krehl (9) and P.H. Sarett (10) conclude that when determining nicotinic acid by bioassay, Leucorostoc mesenteroides should be employed in estimating only free nicotinic acid, since Lactobacillus arabinosus employs the bond types of nicotinic acid amide, nicotinuric acid and co-enzyme I in the same way as free nicotinic acid.

We consulted the results of these tests, and in order to estimate the amount of free and bonded nicotinic acid and the ratio of the contents, we employed both strains and conducted the tests whose results are shown in Table 3. We found that all eight strains produced 0.17 - 2.91 γ/ml of free nicotinic acid and from the estimated values of two types of lactic acid bacteria we found that the bond types produce hardly any nicotinic acid in the culture filtrates. Thus, the production of nicotinic acid in these bacteria groups which do not require thiamine was clear, but when piricularia oryzae, which does not require thiamine, was cultivated in a complete synthetic culture medium to which thiamine was added, we obtained the results shown in Table 4 on testing whether or not nicotinic acid is produced.

According to these results, strains such as the Institute of Agricultural Sciences' 5420, 5514, 5517 and 5526 produce more than one γ/ml, and the other strains produce more than 0.11 γ/ml of nictinic acid. All strains employed produced nicotinic acid, but we believe that no bond types were produced at all. An interesting fact is that the production of about 0.1 to 2.9 mg/l of nicotinic acid is probably due to the piricularia oryzae requiring at least 1 mg/l of thiamine. The strains in which thiamine can be substituted by nicotinic acid probably require an identical amount of nicotinic acid as the others do thiamine.

Table 5 shows the results of estimating the amount of nicotinic acid produced in the culture filtrate and the weight of the mycelia which were weighed at different times, for strain No. 1 of the Agriculture Faculty of Hokkaido University, and strain S0-homo which produces a relatively large amount of nicotinic acid. Both strains No. 1 and S0-homo produce very little nicotinic acid during the period of accelerated growth of mycelia, but at the end of the growth period it is produced rapidly.

TABLE 6. CLASSIFICATION OF MYCELIA OF PIRICULARIA ORYZAE



Legend:

(1) Group with thiamine as essential element; (2) group which grows without thiamine; (3) group in which nicotinic acid cannot be substituted for thiamine; (4) group in which nicotinic acid can be substituted for thiamine; (5) group requiring biotin; (6) group which grows

without biotin; (7) nitric acid reducibility (-) group; (8) nitric acid reducibility (+) group; (9) type 11; (10) type 12; (11) type 13; (12) 5527 (close to CII); (13) group which does not employ $NaNO_2$ as nitrogen source; (14) group which employs $NaNO_2$ as nitrogen source; (15a) inulin (+); (15b) inulin (-); (15c) inulin (+); (15d) inulin (-); (16) sorbose; (17) group employing tryptophane as nitrogen source; (18) group which does not employ tryptophane as nitrogen source; (19) 1. numbers in ( ) refer to classification of National Institute of Agricultural Sciences. Of these, those marked with * are results of 1955. the others are results of 1956. 2. 5518 is not well understood.

As shown above, the fact that there is a considerable difference in the amount of thiamine required and that there are groups in which nicotinic acid can be used in place of thiamine provides an important clue to classification and for this reason, as shown in Table 6, we have revised the classification table given in Report 10 (No. IV). As is clear from Table 6, the nine strains which do not require thiamine can further be divided into two groups according to difference in requirement of biotin (types 12 and 13). We classified as type 11, those groups in which nicotinic acid can be substituted for thiamine. The other groups are as in Report 10 (No. IV).

Summary.

We obtained the following results using ~~47~~ strains of piricularia oryzae ~~which~~ were cultivated in a thiamine-free synthetic culture medium, and in a synthetic medium to which nicotinic acid was added.

~~(1)~~ We established that nine strains reproduced rather well in a thiamine-free synthetic medium, and ~~that~~ these strains produce 0.17-2.91 γ/ml of nicotinic acid in the culture filtrates. The other strains require thiamine.

~~(2)~~ It was shown that there is excellent growth of piricularia oryzae in general in a complete synthetic medium to which thiamine has been added. Also, eight strains developed relatively well in a medium to which nicotinic acid was added in place of thiamine, and ~~we~~ established that nicotinic acid can be employed here as a substitute for thiamine.

~~(3)~~ We cultivated strains which require thiamine in a complete culture medium to which thiamine was added, and found that from 0.11 to 2.64 γ/ml of nicotinic acid was produced.

~~(4)~~ On the basis of the ~~above facts, we have revised our~~ system of biochemical classification of piricularia oryzae into 13 types.

In closing, we would like to express our deep gratitude to professor Hirata Koji of the Pathology Department of the Faculty of Agri-

culture of Niigata University; Assistant Professor Iida Hiroshi of the Applied Microbiology Research Laboratory of Tokyo University, who furnished us with lactic acid bacteria for the bioassays; Professor Tamura Gakuzo of the Faculty of Agriculture of Tokyo University; Professor Kirimura Jiro of the Silkworm Test Station of the Ministry of Agriculture and Forestry; and Professor Miyazawa Shigeru of the Sankyo Takamine Research Laboratory for their assistance in the bioassays. We also thank the National Institute of Agricultural Sciences, the Nagano Agricultural Test Station, the Chugoku Agricultural Test Station, the Agriculture Faculties of Hokkaido and Yamagata Universities, and the Agriculture Faculty of the Tokyo University of Agriculture and Technology for furnishing us with the strains of piricularia oryzae. We are very grateful to the Ministry of Education and Mainichi Shimbun for providing the funds to make this research possible.

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